

This Review is in a thematic series on **Posttranslational Modifications of Cardiac Proteins**, which includes the following articles:

Integration of Troponin I Phosphorylation with Cardiac Regulatory Networks [*Circ Res.* 2013;112:355–366]

Post-translational Modification and Quality Control

Cysteine Oxidative Post-translational Modifications: Emerging Regulation in the Cardiovascular System

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Post-Translational Modifications of Cardiac Myosin Binding Protein C

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Posttranslational Modification and Quality Control

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Abstract: Protein quality control functions to minimize the level and toxicity of misfolded proteins in the cell. Protein quality control is performed by intricate collaboration among chaperones and target protein degradation. The latter is performed primarily by the ubiquitin-proteasome system and perhaps autophagy. Terminally misfolded proteins that are not timely removed tend to form aggregates. Their clearance requires macroautophagy. Macroautophagy serves in intracellular quality control also by selectively segregating defective organelles (eg, mitochondria) and targeting them for degradation by the lysosome. Inadequate protein quality control is observed in a large subset of failing human hearts with a variety of causes, and its pathogenic role has been experimentally demonstrated. Multiple posttranslational modifications can occur to substrate proteins and protein quality control machineries, promoting or hindering the removal of the misfolded proteins. This article highlights recent advances in posttranslational modification-mediated regulation of intracellular quality control mechanisms and its known involvement in cardiac pathology. (*Circ Res.* 2013;112:367-381.)

Key Words: autophagy ■ chaperones ■ chaperone-mediated autophagy ■ deubiquitination ■ neddylation ■ phosphorylation ■ ubiquitin-proteasome system

Genetic mutations and environmental factors, especially those associated with increased stress and pathological conditions, can compromise the integrity of proteins and organelles in the cell, which, if not properly controlled, can be catastrophic to cell function and survival. Hence, the cell has evolved intracellular quality control (QC) mechanisms at protein and organelle levels to minimize the level and toxicity of misfolded proteins and defective organelles in the cell. Inadequate QC is associated with many forms of

heart disease in their progression to congestive heart failure and implicated in cardiac pathogenesis.^{1,2} Hence, improving intracellular QC may potentially become a new therapeutic strategy for cardiac protection. A better understanding of the molecular mechanisms of intracellular QC will facilitate the development of such a novel strategy. Intracellular QC is regulated by transcriptional, translational, and post-translational mechanisms. Posttranslational modifications (PTMs), such as phosphorylation, ubiquitination,

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Non-standard Abbreviations and Acronyms

AAA	ATPase associated with diverse cellular activities
AMPK	AMP-dependent protein kinase
CAMKII	calcium/calmodulin-dependent kinase II
CHIP	C terminus of heat shock protein 70–interacting protein
CMA	chaperone-mediated autophagy
CRLs	cullin-really interesting new gene (RING) ligases
CryAB	α B-crystallin
CSN	COP9 signalosome
DRC	desmin-related cardiomyopathy
DUB	deubiquitinating enzyme
ER	endoplasmic reticulum
HSP	heat shock protein
I/R	ischemia/reperfusion
IPC	ischemic preconditioning
LAMP-2A	lysosome-associated membrane protein 2A
PD	Parkinson disease
PFI	proteasome functional insufficiency
PK	protein kinase
PQC	protein quality control
PTM	posttranslational modification
QC	quality control
Ub	ubiquitin
UPS	ubiquitin-proteasome system

nitrosylation, oxidation, and so on, expand the size of the proteome exponentially and are pivotal in the regulation of protein stability, distribution, and function. Emerging evidence supports a major role of PTMs in regulating multiple pathways of intracellular QC.³ After a brief introduction into the general processes of intracellular QC, this review highlights recent advances in defining the impact of various PTMs on major QC pathways in the cell, especially those with promising roles in cardiac pathophysiology (Table).

An Introduction Into Protein Quality Control

Protein QC (PQC) is a set of molecular mechanisms ensuring that misfolded and damaged proteins are either repaired or removed in a timely fashion, thereby minimizing the toxic effects of misfolded proteins (Figure 1). The QC of proteins targeted for the secretory pathway (ie, proteins passing through the endoplasmic reticulum [ER]) is performed by ER-associated PQC; this involves retrotranslocation of misfolded proteins from ER lumen to the cytosol and degradation of them via ER-associated degradation pathways. ER-independent PQC is responsible for the QC of all non-ER proteins. In both cases, PQC is performed by molecular chaperones and target protein degradation. Chaperones serve as the sensor of misfolded proteins and, in some cases, attempt to repair the misfolding by unfolding/refolding. If the repair fails, the misfolded protein is then termed as a terminally misfolded protein and will be escorted by chaperones for degradation by primarily the ubiquitin-proteasome system (UPS) and perhaps by chaperone-mediated autophagy (CMA). When misfolded proteins escape the surveillance of chaperones and target degradation, they tend to form aberrant

aggregates. The intermediate, highly unstable, soluble species of aggregates are very toxic to the cell.⁴ The small aggregates assimilate into larger ones that are insoluble and perhaps less toxic to the cell. And finally, with assistance from the microtubule transportation system, the small aggregates may be translocated to the microtubule organizing center and fuse with one another to form large inclusion bodies, termed, by some, aggresomes.⁵ The insoluble aggregates and aggresomes are unlikely to be accessible to the proteasome and CMA, both of which can only degrade proteins individually. Hence, the removal of aggregated proteins requires a different mechanism that is capable of bulk degradation of substrates, a role filled by macroautophagy.

Ubiquitin-Proteasome System

UPS-mediated protein degradation consists of 2 main steps: ubiquitination of a target protein molecule and degradation of the ubiquitinated protein by the proteasome. Ubiquitination is by itself an important and common form of PTM. It is a cascade of enzymatic reactions that attach the carboxyl terminus of ubiquitin (Ub) to the ϵ -amino group on the side chain of a lysine residue on either the substrate molecule or a preceding Ub via an isopeptidyl bond. The reaction cascade is catalyzed by the Ub activating enzyme (E1), the Ub conjugating enzyme (E2), the Ub ligase (E3), and sometimes a Ub chain elongation factor (E4). The E3 is most critical because it confers substrate specificity. The proteasome, consisting of a proteolytic core (ie, the 20S proteasome) and regulatory particles (the 19S and the 11S proteasomes), mediates the degradation of polyubiquitinated proteins. Most cellular proteins are degraded by the UPS.⁶ In addition to degrading misfolded proteins for PQC, the UPS participates in the regulation of virtually all aspects of cell functions by timely degradation of normal proteins that are no longer needed. PTMs regulate UPS-mediated proteolysis at multiple levels (see PTMs Regulate the UPS section).

Autophagy

Autophagy mechanisms function by sequestering cytoplasmic components that are degraded by lysosomes. Based on the route via which a substrate enters the lysosomal compartment, autophagy is classified into 3 types: microautophagy, CMA, and macroautophagy. In microautophagy, a small part of the cytoplasm is internalized by invagination of lysosomal membrane and pinched off as a vesicle into the lysosomal lumen wherein it is degraded.

In CMA, a cytosolic protein molecule with a KFERQ motif or KFERQ-like motif is recognized and specifically bound by heat shock cognate 70 and cochaperones, targeted to lysosomal membrane protein (LAMP) 2A, and unfolded and translocated into the lysosome lumen via LAMP-2A.⁷ CMA delivers substrate protein molecules to lysosomes one by one; hence, like the UPS, CMA is capable of target degradation of misfolded proteins. Approximately 30% of cytosolic proteins contain the KFERQ motif in their primary sequences, not including those potentially generated by PTMs.⁷ The KFERQ motifs of many proteins in their native forms are likely buried in the interior, incapable of triggering

Table. Examples of Posttranslational Modifications in Intracellular Quality Control

Targets	PTMs	Regulating Enzymes	Biological Function	References
Chaperones				
HSP90	Acetylation at K294		Suppress substrate binding	24,25
	Nitrosylation at C597		eNOS activation	26,27
	Phosphorylation		Alter substrate binding	30,31
CryAB	Phosphorylation at S59	MAPKAP-K2	Inhibit apoptosis	32,33
HSP27	Phosphorylation		Exacerbate I/R injury	38
Ubiquitin ligases				
CRLs	Neddylation		Facilitate the assembly of CRLs	43
CHIP	Mono-ubiquitination	Ube2W/Ataxin-3	Promote E3 ligase activity	51
Parkin	Neddylation		Increase E3 ligase activity	54,55
UPS substrates				
I κ B	Phosphorylation at S32 and S36.	IKK	Form a phospho-degron	59
Chk1	Phosphorylation at S345	ATR	Expose the degron for ubiquitination	60
Oxidized proteins	Oxidative modification		Generate new degrons	41
p27 ^{Kip1}	Phosphorylation at S10		Translocate to cytoplasm for degradation	61
Proteasome subunits				
20S subunits	Phosphorylation	PKA/PP2A	Increase proteasome activities	67
Rpt6	Phosphorylation at S120	PKA/CAMKII	Increase proteasome activities	68,69,71
α 7	Phosphorylation at S243 and S250.	CK-II	Increase the interaction of 19S-20S	74
Rpn10	Polyubiquitination		Degradation of Rpn10	
Rpn10	Mono-ubiquitination at K84	Rsp5/Ubp2	Reduce proteasome proteolytic function	78
α 1, α 2, α 4	Oxidative modification		Impair proteasome activities	79
Rpt3, Rpt5	Oxidative modification		Impair proteasome activities	80
Autophagy				
Beclin-1	Phosphorylation at T119.	DAPK	Dislodge BCL-2 and activate autophagy	98
	Ubiquitination at K117	TARF6/A20	Unknown	99
BCL-2	Phosphorylation at T69, S70, S87	JNK1/ERK1/2	Disrupt BCL-2/Beclin-1 interaction; activate autophagy	102,103
ULK1	Phosphorylation at multiple sites	AMPK	Activate autophagy	107–109
ATG7	Acetylation	p300/SIRT1	Suppress autophagy	118,119

AMPK indicates AMP-dependent protein kinase; ATR, ataxia-telangiectasia- and rad3-related; CAMKII, calcium/calmodulin-dependent kinase II; CHIP, C terminus of heat shock protein 70-interacting protein; CRL, cullin-RING ligases; CryAB, α B-crystallin; DAPK, death associated protein kinase; eNOS, endothelial NO synthase; HSP, heat shock protein; IKK, I κ B kinase; MAPKAP-K2, mitogen-activated protein kinase-activated protein kinase 2; PK, protein kinase; PP2A, protein phosphatase 2A; PTM, posttranslational modification; and UPS, ubiquitin-proteasome system.

CMA; however, misfolding, partial unfolding, or, in some case, conformational changes resulting from PTMs can all potentially expose the CMA-targeting motif and trigger CMA.⁸ Therefore, CMA may potentially play an important role in PQC by selectively targeting misfolded proteins with a KFERQ motif to the lysosome. CMA activities are reduced during aging due mainly to decreases in LAMP-2A. Restoration of CMA by overexpressing LAMP-2A in livers improves the ability of hepatocytes to handle protein damage and preserves liver function in aged mice.⁹ Inhibition of CMA leads to accumulation of oxidized proteins and protein aggregates in a cell and renders the cell more vulnerable to various stressors.¹⁰ CMA is constitutively activated in cells with impaired macroautophagy.¹¹ Several neurodegenerative disease-associated proteins, in their wild-type form, are degraded by CMA, but their mutant forms often impair CMA.^{12,13} Chronic exposure to a high-fat diet or acute exposure to a cholesterol-enriched diet both were shown to inhibit CMA via destabilizing LAMP-2A on lysosomal

membrane,¹⁴ similar to aging-associated CMA decline. The role of CMA in cardiac pathophysiology remains to be explored.

Macroautophagy delivers bulky cytoplasmic materials, including organelles, into lysosomes via formation of a double-membrane bound vesicle known as an autophagosome for fusion with, and degradation by, the lysosomes. Self-digestion of a portion of cytoplasm provides energy and essential amino acids for the synthesis of important proteins; hence, macroautophagy is essential for cell survival during nutrient deprivation. Macroautophagy is the most studied type of autophagy, and in the past several years it has attracted extensive attention. Manipulating macroautophagy has been proposed to treat certain human diseases. Macroautophagy can selectively degrade defective organelles, such as depolarized mitochondria, as well as aberrant protein aggregates; hence, macroautophagy is considered a major player in both organelle QC and PQC in the cell (see PTMs Regulate Macroautophagy section).

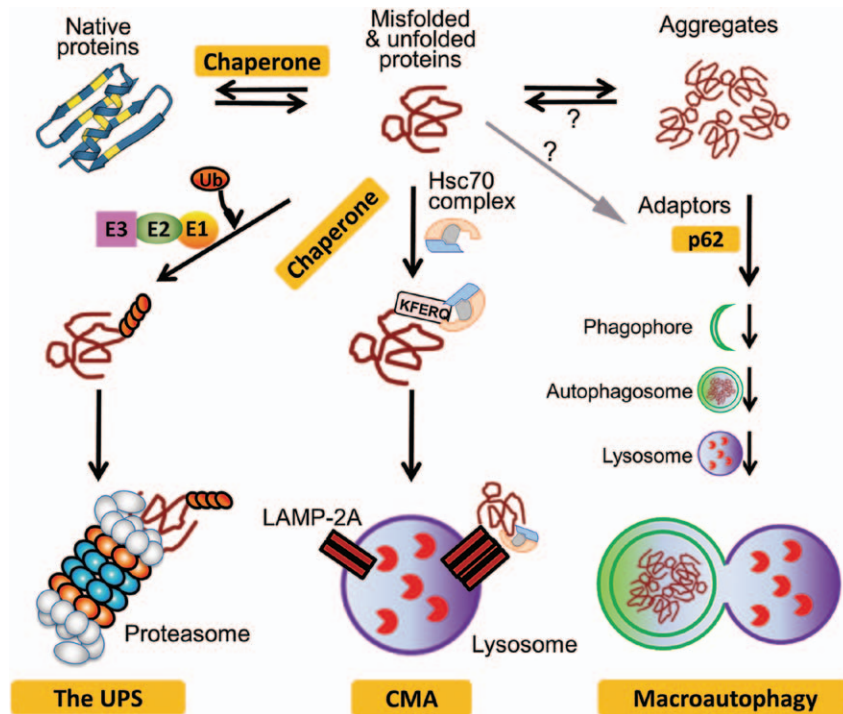


Figure 1. An illustration of protein quality control in the cell. Chaperones help fold nascent polypeptides, unfold misfolded proteins and refold them, and channel terminally misfolded proteins for degradation by the ubiquitin-proteasome system (UPS) or chaperone-mediated autophagy (CMA). When escaped from targeted degradation, misfolded proteins form aggregates via hydrophobic interactions. Aggregated proteins can be selectively targeted by macroautophagy to, and degraded by, the lysosome. hsc indicates heat shock cognate 70; and LAMP-2A, lysosome-associated membrane protein 2A.

Mitochondrial PQC

At the organelle level, the removal of defective mitochondria by macroautophagy (mitophagy) represents an important layer of QC mechanism for cell survival and cell functioning. At the suborganelle or molecular level, proteostasis within mitochondria is critical to the normal functioning of individual mitochondria. The cytosolic UPS might play a role in target degradation of misfolded proteins in the outer membrane of mitochondria, but the interior compartments of mitochondria do not seem to contain the UPS.¹⁵ The refolding of unfolded polypeptides imported from the cytosol, the folding of nascent polypeptides synthesized within mitochondria, and the assembly of stoichiometric protein complexes are monitored by a mitochondrial PQC system localized in different mitochondrial compartments. Analogous to cytosolic PQC, the QC of mitochondrial proteins is performed by mitochondrial chaperones (eg, mitochondrial heat shock protein [HSP] 70 and HSP60) and proteases. In mitochondria, multiple proteases are responsible for the degradation of misfolded mitochondrial proteins. Most known mitochondrial proteases belong to the ATPase associated with diverse cellular activity (AAA) family, including Lon and Clp protease complexes of the matrix, as well as the 2 AAA proteases housed in the inner membrane.¹⁵

The research into cardiac mitochondrial PQC is just beginning to emerge. Lau et al¹⁶ reported that oxidative damage reduced the proteolytic capacity of cardiac mitochondria and decreased substrate availability for mitochondrial proteases. Results of this study further suggested that the 20S proteasome may contribute to cardiac mitochondrial proteostasis by degrading specific substrates of the mitochondria.¹⁶ Further investigation into the physiological and pathophysiological significance of mitochondrial PQC in the heart is well-justified, given that mitochondrial dysfunction has been linked to a variety of heart diseases.

Inadequate PQC in Cardiac Pathogenesis

Inadequate QC occurs in diseased hearts and may play an important role in cardiac pathogenesis. Improving QC protects the heart under stress conditions. The accumulation of ubiquitinated proteins and the presence of aberrant protein aggregation in the formation of preamyloid oligomers in a majority of failing human hearts represent compelling evidence for the existence of PQC inadequacy in the progression of common forms of heart disease to congestive heart failure.¹ The pathophysiological significance of PQC inadequacy is best illustrated by cardiac proteinopathy, which is exemplified by desmin-related cardiomyopathy (DRC). DRC, which can be caused by mutations in the desmin, α B-crystallin (CryAB), and several other genes, is featured by the presence of aberrant protein aggregates in myocytes.¹⁷ Inadequate PQC as represented by proteasome functional insufficiency (PFI) is associated with DRC. Recent experimental studies have demonstrated that PFI plays a major pathogenic role in DRC.² Similarly, PFI was recently unraveled in mouse hearts with acute regional ischemia/reperfusion (I/R) and demonstrated to be an important mediator of myocardial I/R injury.^{2,18} Cardiac expression of chaperones, such as HSPs, is often upregulated with stress, such as I/R or pressure overload, and overexpression of HSPs is cardioprotective under the stress conditions.^{19–21} This suggests chaperone functional inadequacy during the stress condition. Impaired macroautophagy has been observed in I/R hearts, and enhancing macroautophagy has been shown to protect I/R hearts in a large animal model.^{22,23} Hence, improving QC in the cell by increasing chaperones, enhancing UPS-mediated degradation of misfolded proteins, or upregulation of selective autophagy is a potentially novel therapeutic strategy to protect the heart against proteotoxic stress.

PTMs Regulate Chaperone Functions in PQC

A complex network of molecular chaperones promotes efficient protein folding, neutralizes protein damage, and limits protein misfolding and aggregation. Molecular chaperones are defined as proteins that facilitate the following: the folding or unfolding of individual proteins, and the assembly or disassembly of macromolecular complexes, but are not permanent constituents of those complexes. Chaperones play a major role in *de novo* protein folding, in stabilizing unfolded, newly synthesized proteins, and in preventing aberrant interactions, protein misfolding, and aggregation. Chaperones also function in the unfolding of proteins for translocation across membranes of organelles. Chaperone function is important for restoring proteostasis. Chaperones help traffic terminally misfolded and aggregated proteins for degradation by the UPS or autophagy.^{6,24} Many chaperones are HSPs, derived from the fact that cellular stresses, including heat shock, cause protein denaturation, misfolding, and aggregation.

Heat Shock Protein 90

HSP90 is a highly abundant chaperone that uses ATP hydrolysis to facilitate the efficient folding of proteins involved in signal transduction, receptor maturation, and protein trafficking.²⁵ The diverse functions that HSP90 influences rely on the >20 cochaperones, >40 kinases, and receptors it interacts with. HSP90 function is modulated by several PTMs, including acetylation, nitrosylation, and phosphorylation. Acetylation of HSP90 on Lys294 inhibits the binding of cochaperones and client protein.^{26,27} Nitrosylation of Cys597 of HSP90 β inhibits endothelial NO synthase activation.^{28,29} Sites of HSP90 phosphorylation may inhibit (Ser225 and Ser254) or enhance client protein binding (Tyr300).^{30,31} Phosphorylation of cochaperones also influences the function of HSP90.³² Thus, HSP90 chaperone function is regulated by its PTMs and interaction partners.

Heat Shock Protein 70

The HSP70 family consists of 6 members that rely on ATP hydrolysis for activity. HSP70 is responsive to a host of cardiac stresses, such as I/R, and hypertrophic stimuli, including angiotensin II delivery, aortic banding, isoproterenol administration, and swimming exercise.²⁴ HSP70 also interacts directly with histone deacetylase 2 and is necessary for histone deacetylase-dependent hypertrophy.³³ However, few studies exist on the role of PTMs on HSP70 chaperone function.

Small HSPs

HSP22, HSP27, and CryAB are small HSPs that respond to stress. CryAB functions as a chaperone for desmin and perhaps myofibrillar proteins. Mutations in CryAB result in desmin-related myopathies, characterized by protein misfolding and aggregation, suggesting that CryAB chaperone-like activity plays an important role in maintaining cardiac structure and function. CryAB may also play a protective role through inhibition of cardiac hypertrophic signaling in response to pressure overload.²⁰ CryAB is phosphorylated at 3 residues (Ser19, Ser45, and Ser59) with stress. A phospho-mutant CryAB^{S19A, S45A, S59E} that mimics phosphorylation of CryAB at residue 59 protects against osmotic and ischemic stresses and stabilizes mitochondrial membrane potential, *in vitro*.^{34,35}

Thus, phosphorylation may affect the chaperone function and cardioprotective actions of CryAB.

HSP27 expression protects against doxorubicin and lipopolysaccharide-induced cardiac dysfunction.^{36,37} HSP27 is phosphorylated at 3 residues (Ser15, Ser78, and Ser82) in response to ischemic models. Phospho-mutant, HSP27^{S15A, S78A, S82A} transgenic mice is protective against I/R injury.³⁸ Therefore, phosphorylation may inhibit HSP27 chaperone-like activity. PQC is regulated by diverse signals acting on chaperones through a host of PTMs to regulate proteostasis. Better understanding of the mechanisms regulating chaperone function may lead to therapies to counter cardiac pathology.

PTMs Regulate the UPS

At multiple levels, PTMs regulate UPS-mediated proteolysis. First, ubiquitination is by itself a PTM crucial for targeting substrate proteins for degradation by the proteasome, as well as for many nonproteolytic fates of the substrate; second, for most substrate proteins, other PTMs are required for their ubiquitination; third, PTMs regulate the process of ubiquitination by acting on Ub ligases; and finally, the assembly and activity of the proteasome are heavily regulated by PTMs.

Ubiquitination Regulates Both Stability and Activity of Substrate Proteins

Ubiquitination regulates not only the stability but also the localization and function of a substrate protein, depending on the length and topology of the Ub moieties. A chain of ≥ 4 Ubs is often required to target the substrate protein for proteasomal degradation. The 7 lysine residues (K6, K11, K27, K29, K33, K48, K63) of Ub can all be used to form polyubiquitin chains, but K48 and K63 are the 2 most commonly used residues. K48-linked polyubiquitination usually targets the modified proteins for proteasomal degradation, whereas K63-linked polyubiquitination often regulates the subcellular distribution, partner protein binding, and the activity of the modified protein.⁶ For example, TRAF6-mediated K63-linked polyubiquitination of Akt (protein kinase [PK] B) promotes Akt membrane translocation and activation.³⁹ Unlike polyubiquitination, monoubiquitination usually does not trigger degradation but rather signals for nonproteolytic processes, such as signal transduction and transcription regulation. For instance, monoubiquitination of Smad4 at K519 inhibits Smad4 by impeding interaction with phospho-Smad2, playing an important role in transforming growth factor- β signaling.⁴⁰

Regulation of Ubiquitination by Other PTMs

The initiation of ubiquitination obviously requires direct interaction of the substrate with an active specific Ub E3. The signal in a substrate protein to trigger ubiquitination is named a degron. A degron does not seem to be a single consensus segment of primary sequence but rather a conformational signal that mediates the binding of Ub E3 to the substrate. The degron of a native protein often is immature or inaccessible to its specific Ub E3. PTMs of substrates and E3 ligases are usually required to trigger ubiquitination. A misfolded or otherwise damaged protein may trigger ubiquitination by exposing

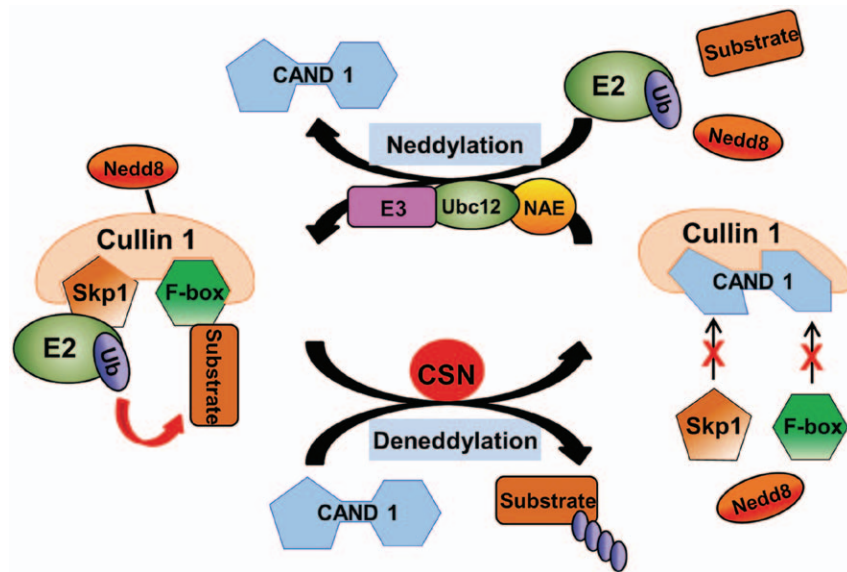


Figure 2. A prevalent model for the regulation of cullin-RING ligases (CRLs) by neddylation-deneddylation. Analogous to ubiquitination, neddylation covalently attaches NEDD8 to a lysine residue of a target protein (eg, cullin). It is catalyzed by NEDD8-activating enzyme (NAE), conjugating enzyme (Ubc12), and presumably specific ligases (NEDD8 E3). As illustrated in the Skp1-Cullin1-Fbox (SCF) ubiquitin (Ub) ligase, cullin neddylation displaces cullin-associated NEDD8-dissociated protein 1 (CAND1), which triggers the assembly of an active CRL complex and brings the adaptor-bound substrate to a close proximity to Ub-charged E2 and allows efficient transfer of the Ub from E2 to the substrate. Deneddylation counters neddylation and is done by deneddylases. The COP9 signalosome (CSN) is the deneddylase responsible for cullin deneddylation. Cullin deneddylation triggers the disassembly of the CRL-substrate complex, releases ubiquitinated substrates, and recycles NEDD8.

hydrophobicity, exposing a buried degron, or generating a new degron.⁴¹

Ubiquitination can be regulated by other PTMs. This is best exemplified by cross talk between phosphorylation and ubiquitination. Phosphorylation can regulate ubiquitination in at least 3 ways. First, phosphorylation of the substrate produces a phosphodegron, which is a short motif that contains phosphorylated residues and mediates phosphorylation-dependent recognition by a Ub E3. Second, phosphorylation of the substrate brings it to the same subcellular location as its specific Ub E3. Third, phosphorylation of E3 modulates Ub ligase activity.

PTMs Regulate E3 Ligase Activities

The specificity of ubiquitination is conferred by the Ub E3 that recognizes and specifically binds a mature degron on the substrate protein. An E3 enzyme harbors the HECT, the RING, or the U-box domain. A Ub E3 can be a single protein (eg, HECT E3), but it is more often composed of multiple proteins. Several E3s have recently been identified to target misfolded proteins for proteasomal degradation, functioning in ER-associated degradation, ribosome-associated PQC, or PQC in the mitochondrial, cytosolic, and nuclear compartments.⁴²

PTMs can regulate Ub E3s via many mechanisms, including altering their assembly, solubility, stability, subcellular localization, or binding affinity toward E2 enzymes and substrates. Cullin-RING ligases (CRLs) are the largest known class of Ub ligases. As exemplified by the Skp1-Cullin1-Fbox E3 complex (Figure 2), a CRL usually consists of a scaffolding cullin, an E2-interacting RING protein, and a substrate-recognizing subunit (eg, an F-box protein). Using various substrate-specific adaptors and a specific cullin, CRLs control the ubiquitination and thus the stability of a wide spectrum of substrate proteins.⁴³ The assembly of a functional CRL is triggered by the conjugation of NEDD8 to the cullin family proteins via neddylation, a ubiquitination-analogous process that uses distinct NEDD8 E1-E2-E3 enzymes.⁴⁴ The cullin neddylation induces conformational changes in cullins, which displaces cullin-associated NEDD8-dissociated protein 1 and brings the Ub-charged E2 and substrates to a close proximity

to facilitate the transfer of Ub from the E2 to the substrate.⁴³ The conjugated NEDD8 can be removed by a deneddylating enzyme, such as the COP9 signalosome (CSN) and SENP8/NEDP1. The cycling of cullin neddylation and deneddylation is essential for the catalytic dynamics of CRLs (Figure 2). Disruption of the cycling, for instance, by inactivating the CSN resulted in autoubiquitination and subsequent destabilization of the CRL components.⁴⁵

An intact NEDD8 modification system is essential for embryonic development. Genetic deletion of a key component of this pathway led to embryonic lethality in multiple model organisms, including mice.⁴⁴ The importance of neddylation in cardiac PQC is recently revealed by the studies on CSN. The CSN comprises 8 distinct subunits (CSN1 through CSN8). CSN-mediated deneddylation requires the CSN holocomplex composed of all 8 subunits. Cardiac-specific ablation of the *CSN8* gene (*CSN8KO*) in mice accumulated the neddylated forms of cullins and noncullin proteins and impaired UPS proteolytic function in the heart.⁴⁶ *CSN8KO* resulted in cardiac hypertrophy, which quickly progressed to lethal dilated cardiomyopathy in mice.⁴⁶ It remains to be elucidated how *CSN8KO* compromises the UPS function. Interestingly, the accumulation of ubiquitinated proteins and elevated proteasomal activities coexisted in *CSN8KO* hearts, implying a possible defect in the delivery of the ubiquitinated substrates to the proteasome.

The mammalian protein C terminus of HSP70-interacting protein (CHIP) is a U-box domain-containing cytosolic PQC E3 ligase that facilitates the degradation of misfolded proteins with the help of chaperones HSP70 or HSP90.⁴⁷ In the heart, CHIP has a strong cardioprotective effect, as demonstrated by inhibition of apoptosis after I/R injury,⁴⁸ angiotensin II-induced cardiac remodeling,⁴⁹ and myocardial infarction.⁵⁰ It was recently found that monoubiquitination of CHIP by an E2 enzyme Ube2w promotes the interaction of CHIP with ataxin-3, a deubiquitinating enzyme (DUB). CHIP-associated ataxin-3 promotes the efficacy of Ub labeling on CHIP substrates, probably by preventing the lengthy Ub chain extension on CHIP substrates. Once the substrates have been

polyubiquitinated, ataxin-3 deubiquitinates Ub-CHIP, leading to the termination of CHIP-mediated substrate ubiquitination.⁵¹ Therefore, modulation of monoubiquitination of CHIP by the pairing of Ube2w and ataxin-3 regulates the transition of the E3 ligase activity between active and inactive state.

Parkin is a well-characterized RING Ub ligase. Mutations of the *Parkin* gene cause a common familial form of Parkinson disease (PD), likely because of parkin loss of function. Parkin plays a crucial role in mitochondrial QC (see UPS-Autophagy Cross Talk section). Many PTMs modify parkin Ub ligase activity. For example, S-nitrosylation of parkin may contribute to pathogenesis of PD by inhibiting parkin Ub ligase activity likely via increasing autoubiquitination of parkin.^{52,53} Neddylation of parkin enhances its interaction with Ub E2, as well as its substrates, increasing parkin Ub ligase activity.^{54,55} Although loss of parkin function has been associated with neuronal and mitochondrial dysfunction,^{56,57} the role of parkin in the heart has just begun to be unveiled.⁵⁸

PTMs of Substrates Regulate Recognition and Binding of Ub E3 Ligases

The discovery that phosphorylation of a substrate protein can create a phosphodegron represents a major insight into inducible UPS-mediated protein degradation. I κ B (inhibitor of nuclear factor κ B) is the best example. Phosphorylation of Ser32 and Ser36 of I κ B by I κ B kinase generates a phosphodegron (DpSGLDpS) that is recognized by F-box protein β -TrCP in the Skp1-Cullin1-Fbox type of Ub E3 complex and targets I κ B for ubiquitination and degradation, playing a critical role in NF κ B activation.⁵⁹ A second scenario is that phosphorylation of a residue outside of the degron induces a conformational change that exposes a degron. For instance, Ser345 phosphorylation exposes a phosphate-free degron in the regulatory region of Chk1, which facilitates its degradation by the UPS.⁶⁰ Instead of promoting ubiquitination, phosphorylation can also prevent a substrate from being recognized by its E3.⁵⁹

Besides phosphorylation, many other PTMs may also regulate ubiquitination at the substrate level. For example, acetylation or sumoylation may compete with ubiquitination for the same lysine residue in the substrate proteins. Cross-linking and conformational changes induced by oxidation may conceivably create degrons de novo and thereby activate UPS-mediated degradation of oxidized proteins.⁴¹

Spatial separation of Ub E3 ligases from their substrates can be a mechanism by which ubiquitination is regulated. Hence, PTMs may promote ubiquitination by bringing substrates and specific E3 ligases into the same subcellular location. A good example is p27Kip1. Phosphorylation of Ser10 in p27Kip1 triggers its translocation from the nucleus to cytoplasm where p27Kip1 is degraded by the UPS.⁶¹

Deubiquitination

Ubiquitination is countered by deubiquitination. The latter removes Ub from ubiquitinated proteins by DUBs. A search for DUB-specific domains in the human genome identified \approx 100 putative DUB-encoding genes. DUBs are Ub proteases, with the majority belonging to the cysteine family of proteases and the rest being metalloproteases. DUBs of the cysteine

family are further classified into 4 subfamilies, based on their Ub-protease domains: ubiquitin-specific protease, ubiquitin C-terminal hydrolase, Otubain protease, and Machado-Joseph disease protease (MJD). All DUBs of the metalloprotease family have a JAMM (JAB1/MPN/Mov34 metalloenzyme) domain. The function, targets, and regulation of most DUBs are not understood. No reported studies have examined the role of DUBs in cardiac PQC; however, studies from other cells/organs have demonstrated an important role in pathophysiology.

DUBs seem to pair with E3 ligases with a relative specificity to regulate ubiquitination,⁶² analogous to the kinase-phosphatase pairing in modulating phosphorylation. From the point of view of PQC, the DUB-E3 pairing between ataxin-3 and CHIP (see Regulation of Ubiquitination by Other PTMs section) or parkin is of particular interest, because polyglutamine track expansion of ataxin-3 is linked to human MJD and parkin mutations cause PD. MJD and PD are closely related neurodegenerative disease with overlapping clinical and pathological features. A major pathogenic role of impaired PQC has been suggested in most neurodegenerative diseases, including MJD, PD, and Huntington disease. Ataxin-3 is a DUB of the MJD subfamily. Ataxin-3 interacts directly with parkin and suppresses parkin autoubiquitination via likely impeding the efficient charging of the E2 with Ub.⁶³ Interestingly, polyglutamine-expanded ataxin-3 promotes degradation of parkin via autophagy,⁶⁴ which helps explain why parkin protein levels are decreased in the brain of transgenic mice overexpressing polyglutamine-expanded but not wild-type ataxin-3.⁶⁴

There are at least 3 DUBs associated with the mammalian 19S proteasome: Rpn11/POH1, Ubp6/ubiquitin-specific protease 14, and ubiquitin C-terminal hydrolase 37. POH1, a JAMM containing DUB, is a stoichiometric subunit of the 19S proteasome. POH1 is responsible for the removal of the Ub chain and Ub recycling during proteasome-mediated degradation of polyubiquitinated proteins. POH1 removes the Ub chain en bloc by cutting at the base of the Ub chain and frees the substrate for translocation into the 20S proteasome. Hence, inhibition of POH1 would nonspecifically suppress proteasome-mediated degradation of polyubiquitinated proteins. In contrast, ubiquitin-specific protease 14 and ubiquitin C-terminal hydrolase 37 trim the Ub chain from its tip distal to the substrate, therefore shortening the chain rather than removing the chain en bloc. This Ub editing function of DUBs might provide an additional layer of regulation on proteasomal degradation of ubiquitinated proteins. A small chemical compound (IU1) capable of inhibiting ubiquitin-specific protease 14 deubiquitination was recently described for its enhancement of proteasome-mediated degradation of some substrates in cultured cells.⁶⁵ This represents the discovery of the first pharmacological proteasome activator. It will be important to test whether IU1 or alike can enhance proteasome function in animals and whether IU1 can effectively treat disease with PFI.

PTMs Regulate the Proteasome

It is now clear that PTMs regulate both assembly and activities of the proteasome. The identification of PTMs on the proteasome has been greatly expanded by recent advances in functional proteomics.³ In addition to proteolytic cleavage, at least

11 different types of PTMs on proteasome subunits are identified, including phosphorylation, acetylation, sumoylation, ubiquitination, hydroxy-2-nonenal modification, oxidation, glycosylation, poly-ADP ribosylation, O-GlcNAc modification, nitrosylation, and N-myristoylation.³ Phosphorylation at specific amino acid residues has been identified in many subunits of the 20S proteasome isolated from the heart using a combination of elegant proteomic approaches.⁶⁶ The biological effect of these phosphorylation sites and the kinases and phosphatases that regulate these sites remain largely undetermined. Nonetheless, the significance of at least some PTMs has been suggested in cardiac proteasomes by recent studies.

N-terminal Cleavage During Proteasome Assembly

All 3 peptidase subunits ($\beta 5$, $\beta 2$, and $\beta 1$) of the 20S proteasome are synthesized as a precursor form in the cell (ie, pre- $\beta 5$, pre- $\beta 2$, and pre- $\beta 1$). The N-terminal proteolytic processing of these precursors is required to expose their catalytic N-terminal threonine (eg, T76 of pre- $\beta 5$ in yeast, T60 of pre- $\beta 5$ in mice) and to generate the catalytically competent form of these subunits during the final step of 20S proteasome assembly where 2 hemi-proteasomes merge to form a full 20S proteasome. The functional significance of this N-terminal processing of pre- $\beta 5$ has recently been demonstrated in cardiomyocytes and mouse hearts. Transgenic expression of a T60A mutant pre- $\beta 5$ in cardiomyocytes dose-dependently inhibited proteasome chymotrypsin-like activity (the activity normally arises from $\beta 5$) and the overall proteasome proteolytic function.¹⁸

Phosphorylation Regulates Proteasome Assembly and Activities

CAMP-dependent PKA mediates β -adrenergic stimulation in the heart, in which phosphorylation on calcium handling and myofilament proteins by PKA impacts profoundly on cardiac function and cardiomyocyte growth. Interestingly, PKA and protein phosphatase 2A can be copurified with cardiac 20S proteasomes.⁶⁷ Further *in vitro* testing showed that PKA is capable of phosphorylating multiple subunits of the 20S and increasing proteasome activities.⁶⁷ Subsequently, a study using nuclear proteasomes from cultured mammalian-486 cells demonstrates that PKA phosphorylates Ser120 of Rpt6, a 19S proteasome subunit, and this phosphorylation fully accounts for the stimulating effect of PKA on proteasome function in these cells.⁶⁸ The pathophysiological significance of PKA-mediated regulation on cardiac proteasomes is illustrated by 2 more recent reports. Asai et al⁶⁹ reported that PKA activation by either β -adrenergic stimulation of the heart or ischemic preconditioning (IPC) rapidly increases the assembly and activity of the 26S proteasome in canine hearts, and the increased proteasome function may contribute to IPC-triggered reduction of total ubiquitinated proteins in I/R hearts. In a mouse cardiac hypertrophy model induced by chronic infusion of isoproterenol, Drews et al⁷⁰ showed that the activity and assembly of the 26S proteasome are increased, but the caspase-like and trypsin-like activities of the 20S proteasome are depressed in the hypertrophic hearts. More importantly, they showed that the depressed 20S proteasome activity can be restored *in vitro* by activating endogenous PKA.⁷⁰

Calcium/calmodulin-dependent kinase II (CAMKII) regulates proteasome function in neuronal cells. In cultured hippocampal neurons, the positive regulation of proteasome function by neuronal activity depends on CAMKII activity. Overexpression of a constitutive active CAMKII stimulated the degradation of a reporter protein in human embryonic kidney cells and stimulates 26S proteasome activity in hippocampal neurons. CAMKII can directly phosphorylate Rpt6 *in vitro*.⁷¹ More recently, it was shown that CAMKII α phosphorylates Ser120 of Rpt6 and enhances proteasome activity. This study also reveals that Ser286-autophosphorylated CAMKII α s serve as a scaffold to recruit proteasomes to dendritic spines, promoting synaptic activity-induced redistribution of the proteasome in hippocampal neurons.⁷² Notably, CAMKII activation has been shown to simultaneously affect mechanical and electric properties of cardiomyocytes and may play a role in the pathogenesis of arrhythmia and congestive heart failure.⁷³ It will be interesting to determine whether CAMKII regulates proteasome function in cardiomyocytes.

It has been shown in animal cells (eg, COS-7) that casein kinase II can phosphorylate the $\alpha 7$ subunit of the 20S proteasome at Ser243 and Ser250. This phosphorylation seems to be constitutive and shows no effect on the peptidase activity, but it increases the stability of the 20S–19S proteasome complex. A decrease in $\alpha 7$ phosphorylation during interferon- γ stimulation seems to destabilize the 26S proteasome, allowing more 20S proteasomes to form complexes with 11S proteasomes.⁷⁴ Notably, upregulation of 11S proteasomes by overexpression of PA28 α , likely via increasing 11S association with the 20S, facilitates the removal of misfolded proteins in cardiomyocytes and protects cardiomyocytes from oxidative and proteotoxic stresses.^{2,75}

PKN, also referred to as PKC-related kinase 1, is a stress-activated serine/threonine kinase. PKN is activated during myocardial I/R and plays a protective role against I/R injury. The protection seems to be mediated by improving PQC, specifically by increasing CryAB phosphorylation and cytoskeletal translocation, as well as by increasing proteasome activity.⁷⁶ It remains to be determined whether PKN directly phosphorylates proteasomes.

Ubiquitination of Proteasome Subunits

Rpn10/S5a is a 19S proteasome subunit but also exists free in the cytosol. Through its 2 Ub interacting motifs, Rpn10/S5a binds ubiquitinated proteins. It is believed to be a main intraproteasomal receptor for polyubiquitinated proteins. It was found that S5a can be polyubiquitinated by all E3 ligases tested and rapidly degraded.⁷⁷ More recently, monoubiquitination at K84 of yeast Rpn10 has been reported.⁷⁸ Rpn10 monoubiquitination is regulated by Ub E3 Rsp5 and DUB Ubp2. K84-monoubiquitinated Rpn10 shows reduced affinity to polyubiquitinated proteins and decreased proteasome proteolytic function. Interestingly, Rpn10 monoubiquitination decreases on proteolytic stress.⁷⁸ These lines of evidence support a biologically relevant role of Rpn10 monoubiquitination in regulating proteasome function. Ubiquitination on several other proteasome subunits has also been identified in various noncardiac cell types, but their biological function is unknown.³

Oxidative Modifications of the Proteasome

Many pathological conditions increase oxidative stress in the heart. The most common one is myocardial I/R that can occur in both the natural course and therapeutic intervention of ischemic heart disease, open heart surgery, and heart transplantation. It has been demonstrated that cardiac proteasome function is depressed by I/R, and this depression plays an important role in I/R injury.^{2,18,79,80} Hence, it is important to determine the mechanism underlying proteasome impairment during I/R. Reperfusion of ischemic myocardium results in an increase in the production of highly reactive free radicals that can cause oxidative modifications to cellular components and damage them. Indeed, analyses of 20S proteasomes purified from I/R myocardium revealed that the lipid peroxidation product 4-hydroxy-2-nonenal selectively modifies $\alpha 1$, $\alpha 2$, and $\alpha 4$ subunits of the 20S.⁷⁹ More recently, oxidative modification of Rpt3 and Rpt5 is associated with impaired proteasome activity in I/R myocardium. IPC attenuates the modification and proteasome impairment as it diminishes I/R injury, suggesting that protection against oxidative damage to the 19S proteasome is a potential mechanism for IPC to reduce I/R injury.⁸⁰ The 26S proteasome activities in crude myocardial protein extracts were found to be significantly decreased in the failing human hearts compared with nonfailing controls. The reduction of proteasome activity was accompanied by oxidative modification of Rpt5 in the failing human hearts.⁸¹ The sufficiency of oxidative modifications to decrease proteasome function has been suggested by studies showing that exposure of purified proteasomes to oxidants, such as hydrogen peroxide and peroxynitrite, inactivates the proteasome.^{82–84}

It has been shown that oxidized proteins can be degraded by the 20S proteasome in a Ub- and ATP-independent manner.⁸⁵ The 20S is more resistant to oxidative damages than the 26S.⁸² In addition to oxidative modification of proteasome subunits, oxidized proteins as the substrate can impair proteasome function.⁸⁶ Conversely, proteasome inhibition can induce oxidative stress.⁸⁷ Hence, oxidative stress and proteasome malfunction may form a vicious cycle.

PTMs Regulate Macroautophagy

Macroautophagy is upregulated with cellular stress, providing an alternative energy source, recycling macromolecules from digested substrates, and heightening intracellular QC. The structures involved in macroautophagic degradation are well defined. The initial step in macroautophagy is the formation and elongation of an isolation membrane, which encompasses cytoplasmic material for degradation. Once structures are completely engulfed by a double-membrane vesicle, they are called autophagosomes. Autophagosomes travel down microtubules and may fuse with vesicular structures to form amphisomes. Autophagosomes and amphisomes then fuse with primary lysosomes, dumping their contents into the lumen of the autolysosome full of hydrolytic enzymes, which degrades the delivered cargo.

Macroautophagy was shown to be an essential process in mouse development, when *beclin 1*^{-/-} mice died early in embryogenesis.⁸⁸ Subsequent loss of autophagy models demonstrated that macroautophagy was a critical process to survive

starvation; *atg5*^{-/-} and *atg7*^{-/-} mice survived until birth and then died during the neonatal starvation period as a result of autophagic deficiency.^{89,90} *Atg7*-null mice revealed that macroautophagy played a necessary role in PQC, when newborns had organs containing Ub-positive inclusions.⁹⁰ Since then, numerous studies have tried to define the role of macroautophagy in the heart.

Beclin 1

Beclin 1 is a component of the class III phosphoinositide 3-kinase complex of autophagosome nucleation, along with Vps34 and p150, responsible for isolation membrane formation.⁹¹ To circumvent the embryonic lethality of *beclin 1*^{-/-} mice, heterozygous (*beclin 1*^{+/-}) mice were used to study the loss of beclin 1 effects in the heart. Hearts from *beclin 1*^{+/-} mice showed reduced autophagosome levels with starvation and pressure-overload conditions, which showed diminished systolic function after 3 weeks of banding.⁹² Cardiac-specific *beclin 1* transgenic mice were created to study *beclin 1* overexpression in the heart.⁹² Hearts overexpressing *beclin 1* showed increased autophagosome levels with starvation and pressure overload.⁹² However, aortic banding led to cardiac malfunction, ventricular hypertrophy, and chamber dilation in beclin 1 transgenic hearts, relative to controls.⁹² These paradoxical results led to the hypothesis that beclin 1-dependent macroautophagy may function across a continuum where too little or too much may be detrimental.⁹³

Beclin 1 protein levels were upregulated in hearts during reperfusion after ischemia. To evaluate the role of beclin 1 with ischemic stress, *beclin 1*^{+/-} mice were subjected to I/R injury.⁹⁴ Hearts from *beclin 1*^{+/-} mice developed smaller infarct sizes than wild-type mice, demonstrating that the loss of beclin 1 function was cardioprotective against I/R injury.⁹⁴

Many types of stress are associated with the accumulation of ubiquitinated, misfolded, and aggregated proteins, including pressure-overload stress.⁹⁵ These accumulations may be due either to insufficient upregulation of proteasome function or inadequate autophagy. To define the role of beclin 1-dependent macroautophagy in PQC, *beclin 1*^{+/-} mice were crossed mutant (R120G) human CryAB mice. The resulting *hCryAB*^{R120G}/*beclin 1*^{+/-} mice developed heart failure prematurely and accumulated more ubiquitinated proteins than *hCryAB*^{R120G}-expressing hearts.⁹⁶ Therefore, the loss of beclin 1 exacerbates proteotoxic pathology and cardiac dysfunction in *hCryAB*^{R120G} cardiomyopathy.

Simple overexpression of *beclin 1* may not induce macroautophagy. Beclin 1 interacts with host cofactors (ATG14L, UVRAG, BIF-1, Rubicon, Ambra1, high-mobility group box 1, nPIST, VMP1, SLAM, IP3R, PINK, survivin, etc), whose interactions can modulate macroautophagy.⁹⁷ Beclin 1 can be phosphorylated by death-associated PK, on Thr119, which causes the dissociation of BCL-2, permitting macroautophagy.⁹⁸ Beclin 1 is also ubiquitinated (Lys117) by tumor necrosis factor receptor-associated factor 6, which is negatively regulated by DUB A20.⁹⁹ Future work will need to define the dominant posttranslational modifications and interaction partners responsible for activating/inactivating beclin 1-dependent macroautophagy in the heart.

BCL-2

Beclin 1 was identified in cells through its interaction with BCL-2. Both BCL-2 and BCL-XL inhibit beclin 1-dependent

macroautophagy by sequestering beclin 1 away from the autophagy nucleation complex.^{100,101} Phosphorylation of BCL2 (Thr69, Ser70, Ser87) by JNK1 causes BCL-2 dissociation from beclin 1, permitting association with the phosphoinositide 3-kinase complex and macroautophagy.¹⁰² High-mobility group box 1 also promotes the phosphorylation of BCL-2 by extracellular signal-regulated kinase (ERK1/2), activating starvation-induced macroautophagy.¹⁰³

Autophagosome levels in cardiac and skeletal muscle increase with exercise duration.¹⁰⁴ Knock-in mice with *bcl-2* phosphorylation sites mutated (Thr69Ala, Ser70Ala and Ser84Ala; *bcl-2* AAA mice) were used to define the role of beclin 1-dependent macroautophagy with exercise.¹⁰⁴ Skeletal and cardiac muscles from *bcl-2* AAA mice were unable to induce autophagosome levels with starvation or treadmill exercise.¹⁰⁴ The *bcl-2* AAA mice were exercise intolerant, with impaired glucose metabolism.¹⁰⁴ Thus, BCL-2 phosphorylation is required for beclin 1 adaptation to exercise.

AMP-dependent Protein Kinase

AMP-dependent PK (AMPK) is activated with low ATP or cell stress to activate macroautophagy. AMPK phosphorylates tuberous sclerosis complex-2 and raptor, triggering the inhibition of mTORC1.^{105,106} Inhibition of mTORC1 stimulates autophagosome synthesis. AMPK also interacts with and phosphorylates ULK1 (Ser317, Ser467, Ser555, Ser574, Ser637, Ser722, Ser757, Ser777, Ser799).^{107–109} ULK1 (Atg1) forms a kinase complex with ATG13, ATG101, and FIP200 responsible for activating macroautophagy under nutrient-deficient conditions. The ULK1 complex is activated when phosphorylated by AMPK and inactivated by mTORC1-dependent phosphorylation. AMPK also stimulates the nuclear accumulation of FOXO3, increasing macroautophagic gene expression.^{110,111}

Ischemic conditions increase AMPK phosphorylation (Thr172) and autophagosome levels.⁹⁴ Transgenic mice expressing dominant-negative AMPK fail to increase autophagosome levels with ischemia and develop larger infarct sizes than wild-type mice.¹¹² Thus, AMPK stimulates autophagosomal content in response to ischemia and starvation.

ATG5 and ATG7

ATG5 is a necessary component for macroautophagy. When conjugated to ATG12, it participates in isolation membrane maturation. ATG5 is necessary for macroautophagy in the adult mouse heart. Conditional *atg5*-deficient mice caused a loss of cardiac function, dilation, and accumulation of ubiquitinated proteins with pressure overload, suggesting that macroautophagy may be cardioprotective.¹¹³ The lack of sufficiency of *atg5* to increase autophagic function has limited its further study.

ATG7 is a key regulator of autophagosome formation; it serves 2 necessary enzymatic functions: in conjugating ATG12 to ATG5 and in adding a phosphatidylethanolamine to ATG8 (LC3).¹¹⁴ Similar to the loss of *atg5* in vivo, knockdown of *atg7* led to cardiomyocyte hypertrophy in vitro.¹¹³ *Atg7* overexpression in cardiomyocytes is sufficient to increase autophagic flux in fed cells.¹¹⁵ ATG7-stimulated macroautophagy successfully reduces misfolded protein and

aggregates accumulation in cardiomyocytes overexpressing CryAB^{R120G},¹¹⁵ a misfolded protein linked to DRC. *Atg7* overexpression has also been shown to rescue autophagic deficiency in other models, in vitro.^{116,117} ATG7 is acetylated by p300 with starvation.¹¹⁸ The p300 acetyltransferase also acetylates ATG4, ATG8 (LC3), and ATG12, which is associated with decreased autophagosome content.¹¹⁸ Other work shows that NAD⁺ also facilitates the acetylation of ATG7, ATG5, and ATG8 (LC3), which can be deacetylated by SIRT1.¹¹⁹

SIRT1

SIRT1 is an NAD-dependent deacetylase that is upregulated with starvation.¹²⁰ SIRT1 acts as a sensor to detect insufficient nutrition and triggers physiological changes linked to health and longevity.¹²⁰ SIRT1 functions to deacetylate ATG5, ATG7, and ATG8 (LC3).¹¹⁹ SIRT1 also deacetylates FOXO proteins, altering FOXO-dependent expression of macroautophagic genes.¹²¹ SIRT1 deacetylation of FOXO1 increases autophagosome levels in glucose-deprived cardiomyocytes.¹²²

To establish the role of *Sirt1* in vivo, cardiac-specific *sirt1*^{-/-} mice were exposed to I/R, and *sirt1*^{-/-} hearts developed larger infarctions than wild types.¹²³ Conversely, *Sirt1*-overexpression mice developed smaller infarct sizes than controls after I/R.¹²³ Thus, SIRT1 is cardioprotective against I/R injury, although more work is needed to define whether FOXO1 acetylation and macroautophagic function are required.

FOXO1/FOXO3

FOXO transcription factors function in many biological processes, including regulation of the expression of *ATG* genes. FOXO1/FOXO3 are subject to multiple PTMs, including phosphorylation, ubiquitination, and acetylation.¹²¹ FOXO1 acetylation is modulated by the balance of histone acetylases and deacetylases. Mutation of 3 FOXO1 lysine residues to arginine (Lys262Arg, Lys265Arg, and Lys274Arg) showed that acetylation of FOXO1 was required for macroautophagy. Acetylation of FOXO1 is required for interaction with ATG7.¹²⁴ However, whether acetylated FOXO1 interaction with ATG7 is required for macroautophagy activation remains to be defined.

FOXO3 expression activates LC3 and *Bnip3* expression and increases autophagosome levels in skeletal muscle cells.¹¹¹ Overexpression of constitutively active *FoxO3* (*FoxO3-CA*) in the heart causes a 25% reduction in heart mass.¹²⁵ However, *FoxO3-CA* mice were unable to counter the pathological hypertrophy from pressure overload.¹²⁵ The mechanisms of FOXO PTMs to activate autophagy-related genes remain to be defined.¹²¹

CSN Regulates Autophagy in the Heart

Neddylolation seems to be involved in regulating autophagy. Disruption of deneddylation by CSN8KO in mouse hearts caused accumulation of autophagosomes and reduced autophagic flux, indicating compromised autophagic degradation.¹²⁶ The autophagic dysfunction was at least partially because of a defect in the fusion of autophagosomes with lysosomes. Downregulation of Rab7 in CSN8KO hearts was likely attributed to the defect.¹²⁶ Given the critical role of the CSN

in regulating both the UPS and autophagy, an intact NEDD8 system may be essential for cardiac PQC. Interestingly, suppression of neddylation by MLN4924, a potent inhibitor of NEDD8 activating enzyme,¹²⁷ activated autophagy in cancer cells, which seemed to be a survival response.¹²⁸ MLN4924-induced autophagy was accompanied by accumulation of Deptor, an inhibitor of mTOR, and was attenuated by down-regulation of Deptor, indicative of involvement of mTOR signaling. Hence, fine-tuned neddylation and deneddylation may regulate macroautophagy, warranting further investigations.

UPS-Autophagy Cross Talk

It becomes increasingly apparent that the UPS and autophagy cross talk to each other, and this cross talk plays an important role in intracellular QC. The cross talk is best exemplified by ubiquitination-triggered autophagic removal of protein aggregates (aggrephagy) or defective mitochondria (mitophagy).

Polyubiquitination linked by any lysine other than K63 can target a protein for proteasomal degradation in yeast.¹²⁹ K63-linked ubiquitination was shown to promote the formation and autophagic removal of aggresomes from misfolded proteins.¹³⁰ p62/SQSTM-1 may mediate the aggregation and autophagic targeting of polyubiquitinated proteins. Through its C-terminal Ub-associating domain (UBA), p62 binds polyubiquitin chains, preferentially K63-linked Ub chain. p62 polymerizes via its N-terminal PB1 domain. p62 also harbors an LC3-interacting region, which may help recruit the LC3-positive phagophore and promote autophagosome formation.¹³¹ The function of p62 in selective autophagy is regulated by its phosphorylation status. Phosphorylation at Ser403 in p62 UBA domain mediated by casein kinase II increases p62 binding to polyubiquitin chains, enhancing inclusion body formation and efficient autophagic degradation of the polyubiquitinated proteins.¹³² Remarkable upregulation of p62 transcript and protein levels, as well as macroautophagy, was observed in mouse hearts overexpressing misfolded proteins.¹³³ In cultured cardiomyocytes overexpressing misfolded proteins, p62 knockdown by small interference RNA decreases aggresome formation, autophagosome marker LC3-II, and cell survival.¹³³ In macroautophagy impaired mice, p62 mediates the formation of Ub-positive inclusion body, but its impact on cell survival seems to be cell type-dependent.¹³⁴ In addition, p62 seems to play a role in mediating autophagic activation by PFI and in chronic autophagic inhibition induced impairment of degradation of ubiquitinated proteins by the proteasome.¹³⁵ The role of p62 in cardiac PQC remains to be investigated *in vivo*.

Given that defective mitochondria can endanger the cell by, for example, increasing production of reactive oxygen species and releasing cell death promoting factors, timely removal of defective mitochondria is an integral part of cytoplasmic QC. The removal of damaged mitochondria is performed primarily by mitophagy. Mounting evidence suggests a critical role of the UPS in triggering mitophagy. In the best-characterized pathway for mitophagy, mitophagy is mediated by PTEN-induced putative PK 1 and E3 ligase parkin. PTEN-induced putative PK 1 accumulated on damaged mitochondria recruits cytosolic parkin to the depolarized mitochondria.¹³⁶ The

mitochondrial parkin then promotes ubiquitination of mitochondrial proteins. Parkin can catalyze the formation of both K48- and K63-linked Ub chains,¹³⁷ both of which seem to be able to trigger mitophagy. According to one model, parkin mediates K48-linked ubiquitination and proteasomal degradation of mitochondrial membrane proteins, including voltage-dependent anion-selective channel protein 1, mitofusin 1 and 2, and other proteins,¹³⁸ which may subsequently facilitate mitochondrial fragmentation, triggering mitophagy.¹³⁹ Supporting this model, removal of damaged mitochondria is blocked by proteasome inhibition.^{139,140} A second model posits that parkin-mediated K63-linked polyubiquitination of mitochondrial substrates recruits p62. p62 then mediates the clustering of damaged mitochondria and subsequent mitophagy in a manner analogous to the aggregation of polyubiquitinated proteins and aggrephagy. However, a recent study shows that p62 is required for parkin-triggered mitochondrial clustering but is dispensable for mitophagy.¹⁴¹ There is also reported evidence that PTEN-induced putative PK 1 and parkin may directly recruit autophagic machinery to the mitochondria.¹³⁷ A critical role for parkin- and p62-mediated mitophagy in cardiac protection by IPC is recently shown by Huang et al.⁵⁸

Concluding Remarks

Existing evidence strongly indicates that PQC becomes inadequate during the progression of many forms of heart disease to congestive heart failure. With virtually no exception, it remains unknown when PQC inadequacy occurs and what is primarily responsible for the inadequacy in a given disease. For instance, defect in the delivery of ubiquitinated proteins to proteolytic chambers is associated with PQC inadequacy in the heart of DRC mouse models.¹⁴² It will be important to determine whether the defect is causative to PQC inadequacy in the DRC hearts and if so, what its molecular basis is.

It is clear that PTMs to either misfolded proteins or the various components of QC machinery, including chaperones, the UPS, and autophagy, impact profoundly on intracellular QC. However, in most cases, the molecular pathways that regulate these PTMs and the significance of the PTMs remain to be defined. Given that a major pathogenic role of inadequate QC in cardiac pathology has been demonstrated, there is a highly compelling rationale to improve our understanding of the regulation of intracellular QC, including the role of PTMs in PQC. Recent advancement in the field of proteomics has made it possible to globally and quantitatively characterize simultaneous changes in PTMs on multiple subunits of the same protein complex at a given condition.³ This is expected to help identify new PTMs and facilitate the quantification of PTM changes in both the substrates and the machinery of QC at baseline and during pathological conditions. Site-specific mutagenesis to abolish or mimic a PTM on the specific residue of a modified protein will continue to be an essential tool to help define the impact of the specific PTM on the function and fate of the modified protein. It will also be important to determine the functional interaction among different PTMs that simultaneously occurred to the target protein. Dissection of the upstream pathways that regulate the PTM will be crucial

to identify novel targets or strategies for developing pharmaceuticals to improve QC in the cell.

It is anticipated that comprehensive investigations into intracellular QC in cardiac physiology and pathology will give rise to new therapeutics to better battle heart diseases, the leading cause of death of humans. On macroautophagy, there is a good body of evidence supporting that activation of macroautophagy improves PQC and thereby protects the heart²³; nonetheless, excessive macroautophagy on certain conditions such as reperfusion may be detrimental.⁹⁴

Some studies, but not others, have shown that pharmacologically induced ubiquitous proteasome inhibition protects against I/R injury and pressure-overloaded cardiac hypertrophy.^{143,144} However, genetically induced moderate proteasome inhibition in cardiomyocytes was recently shown to exacerbate acute I/R injury in mice.¹⁸ Furthermore, administration of bortezomib, a proteasome inhibitor, to multiple myeloma patients can cause reversible heart failure.¹⁴⁵ Conversely, recent genetic studies reveal that proteasome function enhancement in the cardiomyocytes of diseased hearts can slow down the progression of a bona fide cardiac proteinopathy and minimize acute I/R injury in mice.² Hence, it is envisioned that enhancing proteasome proteolytic function may be a potential new strategy to treat heart diseases with increased proteolytic stress. Unfortunately, only one compound (IU-1) has been shown in cultured noncardiac cells to enhance proteasome function.⁶⁵ It is unclear whether IU-1 functions as a proteasome activator in vivo. Therefore, research aiming at facilitating the development of proteasome activators is urgently needed, and its success will have great impact.

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Disclosures

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